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Sec-Mediated Transport of Posttranslationally Dehydrated Peptides in *Lactococcus lactis*▽†

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Nisin is a lanthionine-containing antimicrobial peptide produced by *Lactococcus lactis*. Its (methyl)lanthionines are introduced by two posttranslational enzymatic steps involving the dehydratase NisB, which dehydrates serine and threonine residues, and the cyclase NisC, which couples these dehydrated residues to cysteines, yielding thioether-bridged amino acids called lanthionines. The prenisin is subsequently exported by the ABC transporter NisT and extracellularly processed by the peptidase NisP. *L. lactis* expressing the *nisBTC* genes can modify and secrete a wide range of nonlantibiotic peptides. Here we demonstrate that in the absence of NisT and NisC, the Sec pathway of *L. lactis* can be exploited for the secretion of dehydrated variants of therapeutic peptides. Furthermore, posttranslational modifications by NisB and NisC still occur even when the nisin leader is preceded by a Sec signal peptide or a Tat signal peptide 27 or 44 amino acids long, respectively. However, transport of fully modified prenisin via the Sec pathway is impaired. The extent of NisB-mediated dehydration could be improved by raising the intracellular concentration NisB or by modulating the export efficiency through altering the signal sequence. These data demonstrate that besides the traditional lantibiotic transporter NisT, the Sec pathway with an established broad substrate range can be utilized for the improved export of lantibiotic enzyme-modified (poly)peptides.

Improved variants of therapeutic peptides have great potential in the pharmaceutical market. An important possibility to make new variants is the introduction of dehydrated amino acids into such peptides. Dehydrated residues can enhance or modulate the biological activity of the peptide. For instance, they can play an essential role in peptide activities (35), they can act as inhibitors of biological activities (31, 32), or they may alter and improve signal transmission by interaction with receptor or acceptor molecules (29). Furthermore, dehydrated residues can be starting points for further modifications. A powerful modification is the intramolecular coupling of the dehydrated residue to a cysteine resulting in a (methyl)lanthionine. Cyclization by a thioether ring in the peptide enkephalin led to a drastic improvement of the biostability and in vivo half-life of the peptide (38, 45). Lack of biostability is one of the major limitations for the successful application of therapeutic peptides. Chemical synthesis of therapeutic peptides with dehydrated residues and/or thioether rings is time-consuming and costly. Biological production of such peptides can reduce this cost effectively.

Recently, we have shown that *Lactococcus lactis* can produce and secrete a large variety of dehydrated nonlantibiotic peptides (16, 39) by making use of the lantibiotic enzyme NisB and the ABC transporter NisT of the nisin modification enzyme complex. Four enzymes are involved in nisin biosynthesis: NisB dehydrates serines and threonines in the nisin propeptide. Subsequently, dehydrated residues are stereo- and regioselectively coupled to cysteines in the nisin propeptide by NisC, a feature difficult to achieve by chemical synthesis. Export of the fully modified prenisin occurs via the ABC transporter NisT. Finally, the extracellular peptidase NisP cleaves off the leader, which results in the formation of the active lantibiotic nisin (18, 20, 37, 53). Targeting to and modification by the lantibiotic enzymes depend on the leader peptide (52).

Although NisT is capable of secreting a broad range of modified peptides, not all peptides are transported efficiently (39). Furthermore, the substrate selectivity of NisT has not yet been established. Therefore, other peptide or protein transport systems might be of interest for secretion of modified peptides by *L. lactis*. For the transport of prenisin or nonlantibiotic peptides via NisT, the nisin leader peptide plays an important role. The leader region of type A lantibiotics is highly conserved and does not show the characteristics of the signal peptides used in protein secretion systems (5, 8). Particularly, it has been shown that very short N-terminal extensions of leader peptides do not interfere with the activity of modification enzymes (44, 52). Possibly, an N-terminal extension of the leader sequence with a signal sequence may allow modified peptides to be directed into an alternative protein excretion pathway, thereby increasing the versatility of the modification system for the production of therapeutic proteins.

A well-known universally conserved protein translocation system is the Sec system. The Sec system translocates unfolded proteins across the cell membrane via a protein-conducting pore formed by the SecYEG complex and a molecular motor, the ATPase SecA. Secretory proteins are equipped with an

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Source or reference
Strains		
L. lactis		
NZ9700	*nisABTCIPRKFEG*	23
NZ9000	MG1363 derivative; *pepN*::*nisRK*$^+$	7, 22
LL108	MG1363 derivative; *repA*$^+$ Cm^r	25
B. subtilis 168	*trpC*	24
Plasmids		
pIL253 derived		43
pIL3BTC	pILangBTC derivative; P_{nis} + inverted repeat + *nisBTC*, Cm^r	39
pIL6BTC	pIL3BTC derivative; P_{nis} + *nisBTC*, Cm^r	This study
pILhpB	pIL3BTC derivative; P_{nis} + inverted repeat + *nisB*, Em^r	This study
pILB	pIL3BTC derivative; P_{nis} + *nisB*, Em^r	This study
pIL3BC	pIL3BTC derivative; P_{nis} + inverted repeat + *nisBC*, Cm^r	This study
pILBC	pIL3BTC derivative; P_{nis} + *nisBC*, Em^r	This study
pNZ8048 derived		23
pNZE3	P_{nis} + sequence encoding NisA prepeptide, Em^r	19
pNG41nisA	P_{nis} + sequence encoding SP_{Usp45} fused to nisin prepeptide sequence, Cm^r	This study
pNG41tppii	P_{nis} + sequence encoding SP_{Usp45}-leader peptide-ITSISRASVA; Cm^r	This study
pNG41epo	P_{nis} + sequence encoding SP_{Usp45}-leader peptide-YASHFGPLGWVCK; Cm^r	This study
pNG41lhrh1	P_{nis} + sequence encoding SP_{Usp45}-leader peptide-QHWSYGCRPG; Cm^r	This study
pNG41hrh 2	P_{nis} + sequence encoding SP_{Usp45}-leader peptide-QHWSYSLRCG; Cm^r	This study
pNG41vp1	P_{nis} + sequence encoding SP_{Usp45}-leader peptide-ATFQCAPRG; Cm^r	This study
pNG41acth	P_{nis} + sequence encoding SP_{Usp45}-leader peptide-SYSMECFRWG; Cm^r	This study
pNG51nisA	P_{nis} + sequence encoding SP_{YwbN} fused to *nisA* prepeptide sequence; Cm^r	This study
pNG51tppii	P_{nis} + sequence encoding SP_{YwbN}-leader peptide-ITSISRASVA; Cm^r	This study
pNG51lhrh2	P_{nis} + sequence encoding SP_{YwbN}-leader peptide-QHWSYSLRCG; Cm^r	This study
pTPtppii, pTPvp1, pLPacth, and pLPepo	P_{nis} + sequence encoding leader peptide-therapeutic peptide	16

[a] P_{nis} is the inducible *nisA* promoter.

N-terminal signal peptide sequence which functions as a targeting and recognition signal (9, 36). Signal peptides are composed of three domains: a positively charged N domain, a hydrophobic core (H) domain, and a more polar C domain, which contains the cleavage site for signal peptidases. The latter enzymes remove the signal peptide from the translocated protein, resulting in the release of the mature protein. The average length of a Sec signal peptide is 28 amino acids (48, 51). Protein translocation has been extensively studied in *Escherichia coli* and *Bacillus subtilis*, which are gram-negative and gram-positive bacteria, respectively (50). Homologues of SecA and SecYEG are also present in *L. lactis* (3, 17). Azide, a known SecA inhibitor (30, 33), blocks Sec-dependent translocation of proteins in *L. lactis* (10). Since the number and amount of secreted proteins in the medium of *L. lactis* are relatively low, this organism appears to be an ideal host for the detection and purification of exported therapeutic peptides. The major secreted protein in *L. lactis* is Usp45. The signal peptide of Usp45 has been used successfully for the secretion of heterologous proteins (1, 27, 49). The Sec system in *L. lactis* is able to secrete proteins ranging from low (<5 kDa) to high (>160 kDa) molecular mass (10, 26).

In the present study, we show that the dehydration and cyclization of the nisin propeptide still occur when the nisin leader is preceded by the Sec signal peptide of Usp45 (SP_{Usp45}) and that the Sec system of *L. lactis* efficiently secretes dehydrated therapeutic peptides.

MATERIALS AND METHODS

Bacterial strains and plasmids. The host strain, *L. lactis* NZ9000, was used for expression of the modification enzymes and peptides. The modification genes *nisB* and *nisC* were cloned into pIL253-derived plasmids (43). The coding sequences for the signal peptides were N-terminally fused to the coding sequences of the nisin leader and of the modifiable peptide and placed under control of the nisin-inducible promoter of pNZ8048-derived plasmids (22). Strains and plasmids are listed in Table 1.

Molecular cloning. The *nisBTC* genes, the sequence encoding the Sec signal peptide of Usp45, and the *nisA* gene were amplified from chromosomal DNA of *L. lactis* NZ9700 using the Phusion DNA polymerase (Finnzymes, Espoo, Finland). The Tat signal sequence of YwbN (SP_{YwbN}) was amplified from chromosomal DNA of *B. subtilis* 168. Ligation was carried out with T4 DNA ligase (Roche, Mannheim, Germany). Restriction enzymes used for cloning strategies were purchased from New England Biolabs, Inc. For the construction of pNG41nisA, the start codon of *ssUsp45* was cloned into the NcoI site of pNZ8048. Subsequently, the *nisA* gene was fused at the 3′ end of *ssUsp45*. The plasmid pNG41nisA was used as template for construction of the peptide-encoding plasmids. Plasmids were amplified by using primers with a complementary part to the nisin leader and a 5′ overhang, encoding the peptide sequences, and one universal primer, annealing downstream the *nisA* gene of pNG41nisA. This allowed the exchange of the propeptide NisA sequence with another peptide sequence, leaving the leader peptide intact. The linear peptide-encoding plasmid was self-ligated. For construction of the plasmid pNG51nisA, containing the signal sequence of YwbN fused to the prepeptide NisA, and the plasmids pNG51tppii and pNG51lhrh2, the same strategy was used as described above. For the pIL-derived constructs, the following strategy was used. The *nisTC* genes were removed by restriction of pIL3BTC with EcoRI, followed by ligation, leading to pILhpB. The hairpin between P_{nis} and *nisB* was removed by amplification of pILhpB with an antisense primer containing the ribosome binding site (RBS) behind the P_{nis} promoter and a sense primer beginning with the start codon of *nisB*. The pIL3BC construct was made by amplification on pIL3BTC

with an antisense primer annealing behind the stop codon of *nisB* and a sense primer containing the RBS of *nisC* followed by self-ligation and transformation. From plasmid pIL3BC, the hairpin was removed by amplification with the primers mentioned above, resulting in the plasmid pILBC. Electrotransformation of *L. lactis* was carried out as previously described (11) using a Bio-Rad gene pulser (Richmond, CA). Nucleotide sequence analysis was performed by BaseClear (Leiden, The Netherlands).

Growth conditions. *L. lactis* was grown in M17 broth (46) supplemented with 0.5% glucose (GM17) or in minimal medium (12, 39) with or without chloramphenicol (5 μg/ml) and/or erythromycin (5 μg/ml). Cultures were grown on minimal medium after induction with nisin prior to sample preparation for mass spectrometry (matrix-assisted laser desorption ionization–time-of-flight [MALDI-TOF]), Western blot analysis (16), or the antimicrobial assay.

Mass spectrometry. Samples were purified from the medium fraction by ziptip purification (C_{18} ziptip; Millipore). Dehydration of the peptides was confirmed by ethanethiol treatment, which results in a mass increase of 62 Da upon reaction with the dehydrated amino acid (16). Mass spectra were recorded with a Voyager DE PRO MALDI-TOF mass spectrometer (Applied Biosystems). In order to maintain high sensitivity, an external calibration was applied.

Western blot analysis. Polyclonal anti-leader peptide antibodies were raised in rabbits against the peptide H_2N-STKDFNLDLVSVSKKDC-$CONH_2$ coupled via the cysteine to keyhole limpet hemocyanin (16). Peptides, precipitated with 10% trichloroacetic acid (TCA) from supernatant, or lysozyme-treated cell lysates were dissolved in sample buffer and applied on a tricine gel (41). Peptides were transferred to polyvinylidene difluoride Western blotting membrane (Roche) using a Trans-Blot SD semidry transfer cell (Bio-Rad).

Antimicrobial assay. Samples for analysis, cell lysates or TCA precipitates of culture supernatant, were separated on a Tricine gel. After electrophoresis, the gel was incubated twice in a mixture of isopropanol (20%) and acetic acid (10%) and subsequently washed thoroughly several times with demineralized water (2). The gel was overlaid with top agar containing 1/100 of an overnight culture of the indicator, *Lactococcus lactis* strain LL108, and 0.01 mg/ml trypsin to release mature nisin, and zones of inhibition were scored by halo formation.

RESULTS

Modification by NisB and NisC of nisin prepeptide preceded by nonlantibiotic secretion signals. To investigate whether dehydration by NisB and ring formation in the propeptide by NisC occur when the nisin leader peptide is preceded by a signal peptide, the Sec signal peptide (SP_{Usp45}) or the Tat signal peptide (SP_{YwbN}) was N-terminally fused to the prepeptide NisA. The Tat signal sequence of the YwbN protein directs the protein to the Tat pathway of *Bacillus subtilis* (13). The Tat pathway translocates folded proteins across the membrane (36), and it has been suggested that a tertiary structure of the substrate might be a prerequisite (6). The Tat pathway might be an interesting candidate for the transport of (poly)peptides which have a somewhat bulky character caused by the intramolecular lanthionine rings. Since *L. lactis* lacks a Tat pathway, reconstitution of a Tat pathway in *L. lactis* might be considered. Another possibility is expression of a lantibiotic system in *B. subtilis*.

The corresponding plasmids pNG41nisA (with SP_{Usp45}) and pNG51nisA (with SP_{YwbN}), respectively, were coexpressed with and without pILBC, in the absence of NisT, in *L. lactis*. Samples from cultures with and without coexpression of NisB and NisC were compared by Western blotting (Fig. 1A and B) and by an antimicrobial assay (Fig. 1C). Dehydration, catalyzed by NisB, and ring formation, catalyzed by NisC, were determined by means of a bioassay that monitors the antimicrobial activity of correctly modified nisin in cell lysates after trypsin treatment to remove the signal peptide and the leader peptide. Without coexpression of NisB and NisC, a large amount of prepeptide was secreted when the SP_{Usp45} was used to direct the export (Fig. 1A, lane 2). When the prepeptide was preceded by the Tat signal sequence SP_{YwbN}, only a small amount of the NisA prepeptide could be detected in the supernatant (Fig. 1A, lane 4), even though no functional Tat pathway is present in *L. lactis*. The latter is likely due to inefficient SP_{YwbN}-directed transport via the Sec pathway. When NisB and NisC were coexpressed, no significant level of secreted peptides could be detected in the supernatant when SP_{Usp45} or SP_{YwbN} was used to direct the secretion (Fig. 1A, lanes 3 and 5), which excludes lysis as well as export, and no antimicrobial activity, after trypsin digestion, could be detected in the supernatant (data not shown). However, after analysis of the cell lysates on a Tricine gel using an overlay with the indicator strain together with trypsin, peptides remaining in the cell lysates showed significant antimicrobial activity. This activity was observed at a position in the gel that corresponds to the full length of SP_{Usp45}-leader-NisA of 8.3 kDa (Fig. 1C, lane 3) and SP_{YwbN}-leader-NisA of 10.1 kDa (Fig. 1C, lane 5), respectively. Without coexpression of NisB and NisC, no antimicrobial activity was observed (Fig. 1C, lanes 2 and 4). Also in the absence of trypsin, in all mentioned cell lysates, there was no antimicrobial activity (data not shown). These data

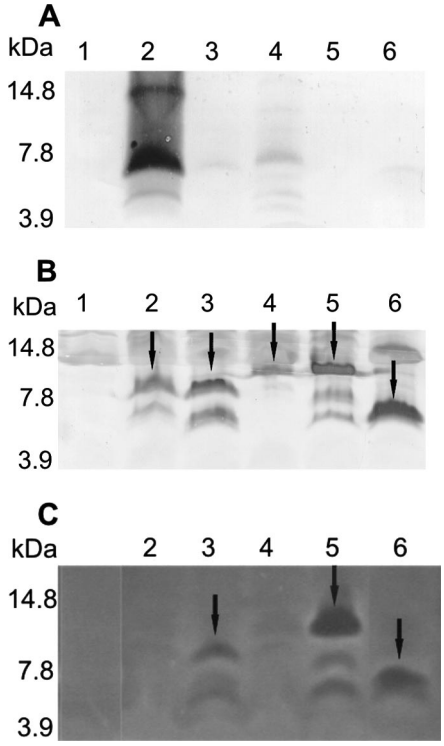

FIG. 1. Modification by NisBC of the NisA propeptide that contains a signal peptide located N-terminally of prenisin. *L. lactis* cells were induced with nisin and grown overnight. TCA precipitates of 1 ml supernatant (A) and of approximately 3.0×10^7 lysed cells (B) were analyzed using Tricine gels and Western blotting with antileader antibodies. By trypsin treatment of the cell extracts on the tricine gel, active nisin could be liberated and detected by an overlay with nisin-sensitive *L. lactis*. The black areas in the picture represent zones with no growth of the indicator strain (C). Lane 1, NZ9000; lane 2, NZ9000(pNG41nisA); lane 3, NZ9000(pNG41nisA pILBC); lane 4, NZ9000(pNG51nisA); lane 5, NZ9000(pNG51nisA pILBC); lane 6, positive control NZ9000(pNZ-E3 pIL3BC). Arrows indicate the full-length products of SP_{Usp45}-leader-NisA, SP_{YwbN}-leader-NisA, and leader-NisA.

TABLE 2. Masses of SP_{Usp45} -directed export of NisB-dehydrated therapeutic peptide variants

Peptide and no. of dehydrations	Sequence[a]	Dehydrated peptide mass ($M + H^+$) in Da[b]	
		Observed	Calculated
TPPII	ITSIS<u>RASVA</u>		
4		3,266	3,267
3		3,284	3,285
2		3,303	3,303
1		3,321	3,321
0		3,339	3,339
LHRH1	<u>QHWSYGCRPG</u>		
1		3,508	3,507
0		3,527	3,525
LHRH2	<u>QHWSY**S**LRCG</u>		
2		3,534	3,535
1		3,552	3,553
0		3,570	3,571
EPO(1–13)	<u>Y**AS**HFGPL**G**WVCK</u>		
1		3,781	3,781
0		3,799	3,799
VP1	<u>**AT**FQ**CA**PRG</u>		
1		3,268	3,267
0		x	3,285
ACTH(1–10)	<u>SYSME**C**FRWG</u>		
1		3,581	3,582
0		3,598	3,600

[a] The amino acids that are part of the potentially active part of the peptide are underlined. Amino acids in boldface correspond to mutations.
[b] Observed masses are of peptides that contain the nisin leader without Met1. Peptide masses with or without dehydration are shown in Da. x, not observed.

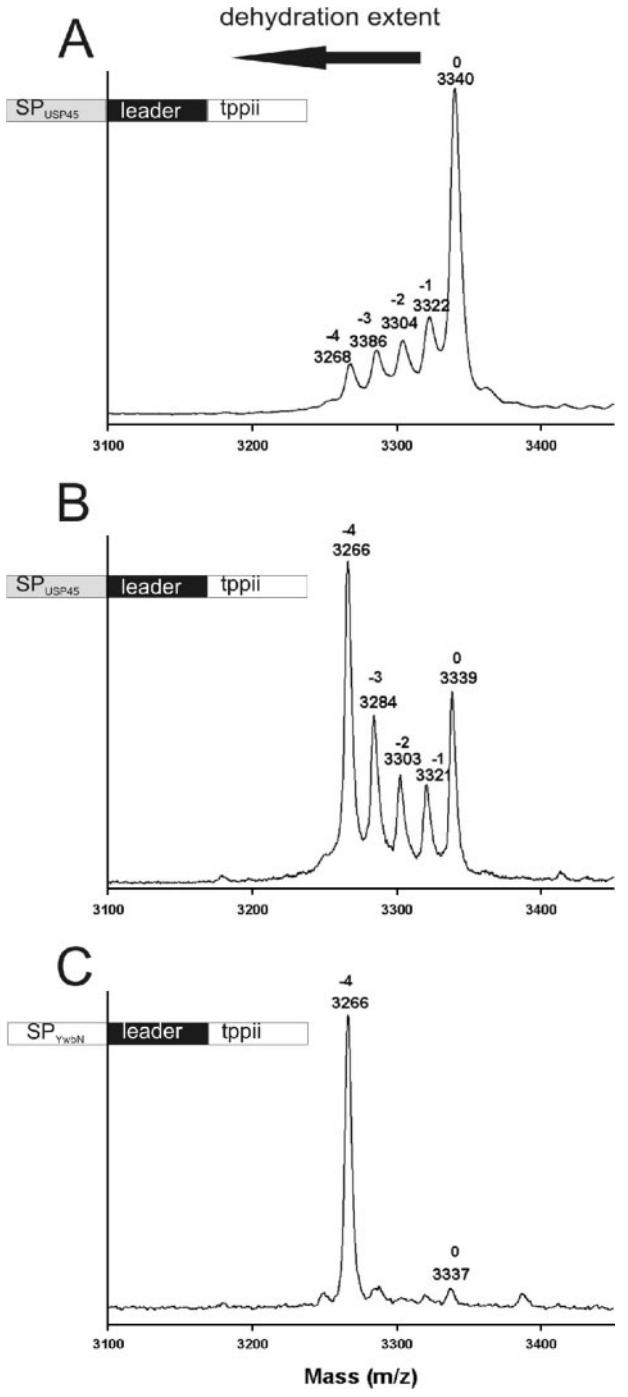

FIG. 2. SP_{Usp45}- and SP_{YwbN}-directed export via the Sec pathway of dehydrated variants of the peptide ITSISRASVA. Culture supernatant was analyzed by mass spectrometry as described in Materials and Methods. (A) Supernatant of *L. lactis* NZ9000(pNG41tppii pILhpB) (with inverted repeat). (B) Supernatant of *L. lactis* NZ9000 (pNG41tppii pILB) (without inverted repeat). (C) Supernatant of *L. lactis* NZ9000(pNG51tppii pILB). The variants dehydrated four times, three times, two times, or one time and those not dehydrated are indicated by −4, −3, −2, −1, and 0, respectively. Intensity of mass spectrometry signals is in arbitrary units and was left out in the mass spectra.

demonstrate that both NisB and NisC are capable of modifying their substrate despite the fact that the leader peptide is preceded by a signal peptide 27 (SP_{Usp45}) or 44 (SP_{YwbN}) amino acids long. Moreover, the absence of extracellular levels of fully modified prenisin suggests that secretion of the fully modified peptide via the Sec pathway is impaired.

Sec-mediated transport of NisB-dehydrated peptides. Recently, we have shown that the ABC transporter NisT of *L. lactis* transports a wide range of dehydrated nonlantibiotic peptides into the culture medium (16). Here, we investigated whether the general Sec pathway of *L. lactis* can be exploited for the translocation of dehydrated peptides, thereby increasing the range and amount of modified peptides and proteins that can be produced and exported. For this purpose, plasmids (pNG41peptide) encoding fusions of the signal peptide of Usp45 (SP_{Usp45}) with the nisin leader and the peptides of interest, including variants of the inhibitor of tripeptidylpeptidase II (TPPII), the luteinizing hormone-releasing hormones (LHRH1 and LHRH2), the erythropoietin (EPO) fragment (positions 1 to 13), vasopressin (VP1), and an adrenocorticotropic hormone (ACTH) fragment (positions 1 to 10), were coexpressed with a pIL-derived plasmid carrying *nisB* (Table 1) in *L. lactis* NZ9000, in the absence of *nisC* and *nisT*.

Of all therapeutic peptides analyzed, dehydrated variants (−18 Da) could be detected in the supernatant by mass spectrometry (Table 2 and Fig. 2A [and see Fig. 5A]). Dehydration

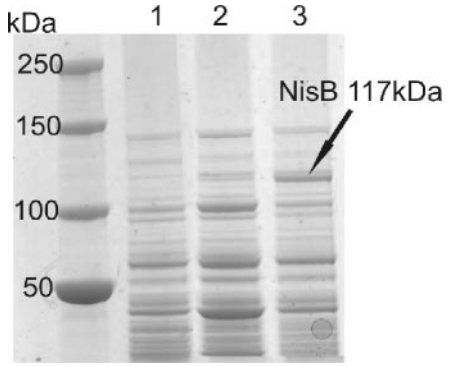

FIG. 3. Increased NisB level following removal of the inverted repeat in front of *nisB*. *L. lactis* cells were induced with nisin and grown overnight. Approximately 3.0×10^7 lysed cells were analyzed by Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis for NisB detection. Lane 1, NZ9000; lane 2, NZ9000 containing pNG41tppii and pILhpB (with inverted repeat); lane 3, NZ9000(pNG41tppii pILB) (without inverted repeat).

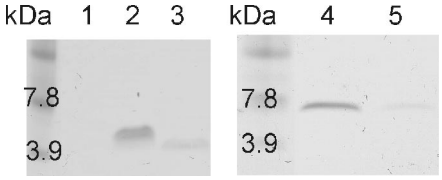

FIG. 4. Comparison of secretion levels of peptides preceded by SP_{USP45} and SP_{YwbN}. The equivalent of 2.5 ml of supernatant was analyzed on a Coomassie-stained Tricine gel. Lane 1, NZ9000(pTPtppii) (no signal sequence, no NisT); lane 2, NZ9000(pILB pNG41tppii) (SP_{USP45}, no NisT); lane 3, NZ9000(pILB pNG51tppii) (SP_{YwbN}, no NisT); lane 4, NZ9000(pIL5B pNG41lhrh2); lane 5, NZ9000(pIL5B pNG51lhrh2). Cultures were induced at an optical density at 600 nm of 0.4, and supernatant was harvested at an optical density at 600 nm of close to 1.0.

of the peptides was confirmed by ethanethiol treatment (data not shown). Without expression of NisB, all peptides were secreted without dehydrations (data not shown). Observed and calculated masses of the modified peptides are summarized in Table 2. These data demonstrate that NisB is still capable of dehydrating the substrate peptides even when a Sec signal peptide (SP_{Usp45}) is fused to the N-terminal part of the leader peptide and that the versatility of the Sec pathway of *L. lactis* can be exploited to secrete a wide range of dehydrated peptides. In particular, the Sec pathway provides a good alternative for the peptides VP1, ACTH, and EPO, which are inefficiently exported by NisT (see Fig. S1 in the supplemental material; compare, respectively, levels of export of different peptides via Sec in lanes 2, 4, and 6 with those of export via NisT in lanes 1, 3, and 5).

Increased peptide dehydration upon overexpression of NisB. We initially used a pIL-derived plasmid with an inverted repeat upstream of *nisB*, as it is encoded in the nisin gene cluster *nisABTCIPRKFEG* on the chromosome of *L. lactis* NZ9700. This inverted repeat results in a partial readthrough by the RNA polymerase into genes downstream of the *nisA* gene, which is thought to yield an optimal stoichiometry of the NisA prepeptide; the modification enzymes, NisB and NisC; and the transporter, NisT (21, 34). After removal of the inverted repeat sequence between P_{nis} and *nisB* on pILhpB, a significant increase in the extent of dehydration of the peptides was observed as shown for the inhibitor of tripeptidylpeptidase II peptide, TPPII (compare Fig. 2A and B). This is likely due to an increased activity caused by elevated expression of NisB as verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie staining of cell lysates (Fig. 3) (also see reference 14).

Improvement of extent of dehydration by using the Tat signal sequence. An intriguing question is whether modulation of the transport efficiency of the peptide influences the extent of dehydration of the exported peptides. Interestingly, we observed that the Tat signal sequence, SP_{YwbN}, can be used to secrete the prepeptide NisA by the Sec system into the supernatant (Fig. 1A, lane 4). However, lower quantities of peptide were exported, which points at an inefficient targeting and/or recognition of SP_{YwbN} by the Sec system. To investigate whether these slower kinetics increase the opportunity of the peptide substrate to interact with NisB, and thereby influence the extent of dehydration, SP_{YwbN} was used to direct dehydrated peptides to the Sec pathway. From the induced cultures of *L. lactis* NZ9000(pNG51tppii pILB) and NZ9000(pNG51lhrhr2 pILB), which thus lack NisT, the supernatant was analyzed by Western blotting and mass spectrometry. As expected, a lower level of secretion was observed when SP_{YwbN} was used instead of SP_{Usp45} to direct the export of the TPPII or LHRH2 peptide (Fig. 4). However, as can be seen on the Coomassie-stained gel, a reasonable amount of peptide was still secreted, even when SP_{YwbN} was used to direct secretion (Fig. 4, lanes 3 and 5). In a control experiment, no secreted peptides could be detected in the supernatant when the peptide was not preceded by a signal peptide together with absence of NisT (Fig. 4, lane 1), a result that excludes lysis. The mass spectra of the more slowly secreted TPPII revealed that the peptide was nearly fully dehydrated (compare Fig. 2B and C). Similarly, the extent of dehydration of LHRH2 peptide was also increased when the peptide was secreted by means of SP_{YwbN} (compare Fig. 5A and B). Furthermore the main peaks detected by mass spectrometry corresponded with the peptides TPPII and LHRH2, nicely processed by the signal peptidase. The amounts of the peptides in the supernatant and their complete processing are consistent with Sec-mediated secretion. These data demonstrate that reduced secretion caused by a poor targeting signal is accompanied by an improved and more complete level of dehydration of the secreted peptides.

Spontaneous ring formation. Cysteinylation of an available cysteine in a peptide gives a mass shift of 119 Da that can be detected by mass spectrometry (Fig. 5A and B). Spontaneous ring formation by coupling of a dehydrated residue to a cysteine in the same peptide sequence prevents this cysteinylation. For instance, no cysteinylation of the double-dehydrated variant of LHRH2 (Fig. 5A and B) or of ACTH was observed in the mass spectra. On the other hand, cysteinylation of the nondehydrated variant or the single-dehydrated variant of LHRH2 was clearly detectable, suggesting spontaneous ring formation in the double-dehydrated variant of these peptides. When a thioether bridge is formed in the LHRH2 peptide (QHWSYSLRCG), it is likely that the arginine becomes protected against cleavage by trypsin. Peptides precipitated from the supernatant of NZ9000 cells transformed with pNG41lhrh2

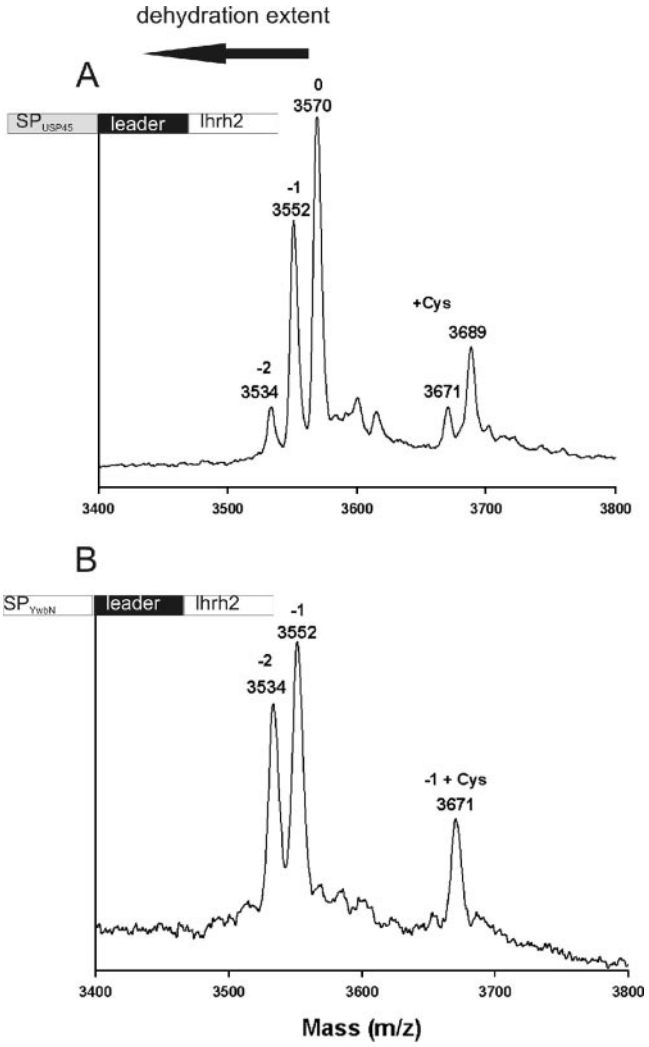

FIG. 5. Enhanced dehydration of LHRH2 when using the YwbN signal peptide compared to the Usp signal peptide. Culture supernatant was analyzed by MALDI-TOF as described in Materials and Methods. (A) Supernatant of *L. lactis* NZ9000(pNG41lhrh2 pILB) (SP_{USP}). (B) Supernatant of *L. lactis* NZ9000(pNG51lhrh2 pILB) (SP_{YwbN}). The peptides dehydrated twice or once and nondehydrated peptides are indicated by −2 or −1 and 0, respectively. Cysteinylation (+Cys) to an available cysteine results in a shift of 119 Da.

pILB or pNG51lhrh2 pILB were treated with trypsin and analyzed by mass spectrometry (see Fig. S2A and B in the supplemental material). The nonmodified LHRH2 peptide from the NZ9000(pNG41lhrh2 pILB) culture was completely digested behind the arginine position. However, the single-dehydrated LHRH2 variants of both cultures were only partially digested, suggesting some degree of ring closure. Moreover, the double-dehydrated variant of the LHRH2 peptide of both cultures was entirely trypsin resistant, consistent with complete ring closure.

DISCUSSION

The general secretion (Sec) pathway of *L. lactis* has been used successfully for the secretion of heterologous proteins. Here, we have investigated whether the Sec pathway can also be used to secrete dehydrated therapeutic peptides. The study demonstrates that NisB-catalyzed dehydration of serine and threonine residues in peptide sequences can still take place even when the nisin leader is preceded by a Sec or Tat signal sequence. Since export is observed in a strain lacking the lantibiotic transporter NisT, it appears that the dehydrated peptide fusion proteins are secreted via the Sec pathway. Some peptides are even secreted to higher levels than those observed with NisT-mediated transport.

To enhance the extent of dehydration of Sec-transported peptides, two successful approaches were followed. First, the dehydration of the secreted peptide could be improved by raising the intracellular concentration of NisB. Thus, it is evident that the NisB/substrate ratio has a considerable effect on the extent of dehydration of the secreted peptide. Lowering the intracellular peptide concentration while raising the NisB concentration will most likely result in an even greater extent of dehydration of the peptide. Second, less efficient targeting and transport also lead to a higher level of dehydration of the secreted peptide. Reduced translocation kinetics might increase the opportunity for NisB to dehydrate the fusion peptide. Indeed, the extent of dehydration improved when the Tat signal instead of the Sec signal sequence was used. Since *L. lactis* does not contain a Tat translocase, the heterologous signal sequence of YwbN of *B. subtilis* most likely targets the peptide to the Sec system. It has been reported before that inefficient, low-level targeting and translocation of a Tat-directed substrate via the Sec pathway can occur (40). In conclusion, in vivo modulation of the enzyme:substrate ratio and/or the efficiency of transport, by modulating the signal sequence, can be valuable approaches to improve peptide dehydration while maintaining an appreciable level of peptide export.

Another important observation is that NisB is functional in the absence of NisT and NisC. Previously, we have demonstrated that NisT functions in the absence of NisB and NisC and is able to transport nonlantibiotic peptides (19). Recently, in vitro activity of NisC, in the absence of NisB and NisT, has been demonstrated (28). When combining these results, we now can conclude that the enzymes of the proposed membrane-associated multimeric lanthionine synthase complex (NisBTC) (15, 42) can function independently. Thus, peptide modification and secretion are intrinsically uncoupled processes.

Furthermore, NisC is still able to cyclize the dehydrated prepeptide NisA when preceded by the Sec signal sequence, as evidenced by the antimicrobial assay. As a result of NisC-mediated modification, secretion of the fully modified prenisin via the Sec pathway appeared to be impossible. The (methyl)-lanthionines in nisin give the peptide a bulky character which probably precludes its export via the translocation pore of the Sec transport system. The dimensions of the solvent-accessible surface of a completely modified nisin are about 2.2 by 2.7 by 4.2 nm (4), whereas molecular dynamics simulations suggest that the monomeric SecY pore has a maximal pore diameter of about 1.6 nm (47). These data therefore suggest that the completely modified nisin molecule is too large to fit in the SecY pore. On the other hand, translocation of peptides with a single lanthionine via the Sec pathway might still be possible as our results with the therapeutic peptides did not establish if ring

formation has occurred intra- or extracellularly. Possibly, fully modified nisin can be translocated via the Tat system. In order to establish such a phenomenon, it will be necessary to reconstitute the Tat pathway in *L. lactis* by introducing a system from another bacterial host, such as *B. subtilis*, or express the nisin system in an alternative host with an endogenous Tat system.

In conclusion, here we show for the first time that at least one completely different transport machinery besides the dedicated lantibiotic transporter NisT can be used for the export of lantibiotic enzyme-modified peptides. Our present findings imply that the Sec system with an established broad substrate range can be used for the export of peptides with dehydrated amino acids, although completely modified prenisin with its multiple thioether rings appears not to be tolerated. The use of the Sec system provides a greater versatility to produce peptides with dehydrated residues followed by in vitro cyclization of the dehydrated residues to cysteines, either chemically or enzymatically. Taken together these findings underline the large potential to apply lantibiotic enzymes for the biotechnological production of modified peptides.

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